Mitochondrial DNA Relationships in an Emergent Pest of Honey Bees: *Aethina tumida* (Coleoptera: Nitidulidae) from the United States and Africa

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Ann. Entomol. Soc. Am. 93(3): 415–420 (2000)

ABSTRACT The hive beetle *Aethina tumida* Murray is a new pest of honey bee colonies in North America. Specimens of *A. tumida* were collected throughout its current range in the southeastern United States, and from several sites in South Africa. A 1018-bp section of the mitochondrial cytochrome c oxidase I gene was amplified and sequenced in 26 beetles collected from Florida, Georgia, South Carolina, and North Carolina, and in 14 beetles collected from seven sites in South Africa. Mitochondrial DNA variation between all *A. tumida* samples was <0.8%, which was still considered within the range expected for a single species. The U.S. samples showed two distinct haplotypes, differing by 6 bp (0.6%). Both haplotypes were found across and within several geographic regions, a result consistent with a single introduction into the United States. However, a broad survey of 151 beetles from their new range revealed significant heterogeneity in haplotype frequencies, perhaps resulting from multiple introductions. Although the data do not allow a precise estimate of the point from which *A. tumida* were accidentally exported from Africa, the close genetic similarity between beetles from the United States and South Africa indicates that studies conducted on beetle physiology, parasites, and pathogens in South Africa will have a direct bearing on populations now found in the United States.

KEY WORDS *Apis mellifera, Aethina tumida,* mtDNA, phylogeny, invasive pest, molecular systematics

INTRODUCED PEST SPECIES frequently show dramatic population growth, resulting in high costs to agricultural systems and other industries, along with damage to natural ecosystems (Pimm 1996). Outbreaks by introduced pests can be mitigated by chemical and biological controls (e.g., Huffaker and Messenger 1997), although such controls generally follow extensive study of pests in their introduced and native ranges. *Aethina tumida* Murray has emerged over the past 2 yr as a serious pest of honey bees, *Apis mellifera* L., in the United States (Elzen et al. 1999). *A. tumida* feed on honey, and pollen, and brood in honey bee colonies, and have been implicated in both colony mortality and an increased tendency by bees to abscond from hives. Curiously, *A. tumida* is of only minor consequence to honey bees and beekeepers in South Africa, the presumed source for beetles now found in the United States. In one survey, only 7% of South African beekeepers listed *A. tumida* as a serious pest, whereas 28% of the respondents considered the greater wax moth to be a serious pest (Buys 1975). Similarly, beekeepers in Uganda reported wax moths to be a more serious pest than hive beetles (Roberts 1979).

Several factors might explain the discrepancy in impact between *A. tumida* in the United States and Africa. First, the honey bee races common in Africa are distinct from those commonly used by U.S. beekeepers. It is conceivable that African honey bees, primarily *Apis mellifera scutellata,* have evolved effective defensive strategies against *A. tumida.* Although comparative data do not yet exist for hive beetles, *A. m. scutellata* workers generally are more aggressive toward invasive pests than are workers of the honey bee race now common to North America (*Apis m. ligustica;* Eischen et al. 1986). Second, different beekeeping practices between Africa and the United States might help to keep *A. tumida* populations in Africa at low levels. Most notably, beekeepers in Africa tend to minimize the volume of honey stored in hives, compared with U.S. beekeepers. This could reduce the ability of *A. tumida* to grow to vast numbers in hives. Third, as a result of their limited introduction into the United States, *A. tumida* might have escaped from important parasites, predators, or pathogens that limit African populations. Release from such enemies has been implicated in the success of other invasive species (Huffaker and Messenger 1997). Finally, *A. tumida* populations now found in the United States might represent a distinct, and more virulent, taxon when compared with those commonly recognized in

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Table 1. Collection records for sequenced samples of *A. tumida* from the United States (by state) and South Africa (SA)

Beetle ID	State	County	Site	Collector	Date
FLDelF16	FL	Volusia	Deland	J. Pettis	Feb. 1999
FLDelG10	FL	Volusia	Deland	J. Pettis	Feb. 1999
FLDelG11	FL	Volusia	Deland	J. Pettis	Feb. 1999
FLHarvB14	FL	Palm Beach	Harvey	R. Harvey	Feb. 1999
FLHarvD13	FL	Palm Beach	Harvey	R. Harvey	Feb. 1999
FLVeroF14	FL	Indian River	Vero Beach	J. Pettis	Feb. 1999
GAHutchF7	GA	Chatham	Hutchinson Island	J. Pettis	Feb. 1999
GARichF11	GA	Chatham	Richmond Hill	J. Pettis	Feb. 1999
GARichF12	GA	Chatham	Richmond Hill	J. Pettis	Feb. 1999
NCAmbAG6	NC	Scotland	AmbroseA	J. Ambrose	Mar. 1999
NCAmbAH1	NC	Scotland	AmbroseA	J. Ambrose	Mar. 1999
NCAmbBH4	NC	Scotland	AmbroseB	J. Ambrose	Mar. 1999
NCLaurD12	NC	Scotland	Laurinburg	D. Hopkins	Feb. 1999
NCLaurE4	NC	Scotland	Laurinburg	D. Hopkins	Dec. 1998
NCLaurE5	NC	Scotland	Laurinburg	D. Hopkins	Dec. 1998
SCCharlE8	SC	Charleston	Charleston	M. Hood	Jan. 1999
SCCharlE9	SC	Charleston	Charleston	M. Hood	Jan. 1999
SCCharlF1	SC	Charleston	Charleston	J. Pettis	Feb. 1999
SCCharlF3	SC	Charleston	Charleston	M. Hood	Mar. 1999
SCCharlG3	SC	Charleston	Wadmalon Island	M. Hood	Mar. 1999
SCCharlG4	SC	Charleston	Wadmalon Island	M. Hood	Apr. 1999
SCDorchG1	SC	Dorchester	Dorchester	L. Motes	Nov. 1996
SCDorchG2	SC	Berkeley	Moncks Corner	B. Carter	Oct. 1997
SCKerG5	SC	Kershaw	Kershaw	S. Taber	Mar. 1999
SCStJoF4	SC	Charleston	St. John's Isle	J. Pettis	Feb. 1999
SCStJoF5	SC	Charleston	St. John's Isle	J. Pettis	Feb. 1999
SAIxopoA9	SA	Durban	Ixopo	H. Shimanuki	Oct. 1998
SANStelB8	SA	Stellenbosch	Niev. Stell.	H. Shimanuki	Oct. 1998
SANStelD2	SA	Stellenbosch	Niev. Stell.	H. Shimanuki	Oct. 1998
SANStelD3	SA	Stellenbosch	Niev. Stell.	H. Shimanuki	Oct. 1998
SANStel12	SA	Stellenbosch	Niev. Stell.	H. Shimanuki	Oct. 1998
SACpPtJ1	SA	Cape Town	Cape Point	M. Allsopp	Apr. 1999
SACpPtJ2	SA	Cape Town	Cape Point	M. Allsopp	Apr. 1999
SARietF17	SA	Pretoria	Rietondale	D. Swart	Feb. 1999
SARietF18	SA	Pretoria	Rietondale	D. Swart	Feb. 1999
SARietH20	SA	Pretoria	Rietondale	D. Swart	Feb. 1999
SARichJ5	SA	Durban	Richmond	M. Allsopp	Apr. 1999
SARichJ6	SA	Durban	Richmond	M. Allsopp	Apr. 1999
SAGtwnJ7	SA	Pt. Elizabeth	Grahamstown	M. Allsopp	Apr. 1999
SAGtwnJ8	SA	Pt. Elizabeth	Grahamstown	M. Allsopp	Apr. 1999
SAPotchJ3	SA	Pretoria	Potchefstroom	M. Allsopp	Apr. 1999
SAPotchJ4	SA	Pretoria	Potchefstroom	M. Allsopp	Apr. 1999

Africa. Molecular-genetic analyses of *A. tumida* from the United States and Africa will be helpful for addressing the latter two hypotheses, and should help guide current and future control strategies directed at the *A. tumida.* Molecular-genetic analyses can also shed light on the timing and magnitude of the original introduction of *A. tumida* into the United States.

Here, we use mtDNA sequences to infer phylogenetic relationships among *A. tumida* and two outgroup taxa, collected across the current range in the United States and from seven putative source populations in South Africa. We follow this with a survey of haplotype variation across multiple states in the United States, as a preliminary step toward estimating the magnitude of the initial introduction of *A. tumida* into the United States, the number of distinct introductions, and the dynamics of hive beetle dispersal since introduction.

Materials and Methods

Biological Material. Adult *A. tumida* were collected directly from honey bee hives in the United States and South Africa, between November 1996 and March 1999 (see Table 1 and Figure 1 for collection information). After collection, beetles were preserved in 95% ethanol before genetic analyses. *A. tumida* were identified to species using published morphological descriptions (Connell 1956, Gillogly 1965) and by comparison to specimens in the USDA–ARS Bee Research Laboratory reference collection. Two additional beetle taxa, a nitidulid beetle in the genus *Colopterus,* and a ladybird beetle in the genus *Hippodamia* (Coleoptera: Coccinellidae) were collected in Beltsville, MD, to be used as outgroup taxa. Physical voucher specimens of the samples used, along with DNA vouchers, are being held at the Bee Research Laboratory, Beltsville, MD.

DNA Extraction, Amplification, and Sequencing. Individual beetles, or beetle abdomens, were dried at 50°C in 1.5-ml microcentrifuge tubes, and ground to a powder using a sealed pipette tip attached to a motorized drill. Five hundred microliters of lysis-digestion buffer (0.01 M Tris pH 7.8, 0.005 M EDTA, 0.5% SDS with 1 mg/ml proteinase K) were added to each sample, which were then vortexed and placed into a

55°C water bath. Samples were incubated for 1 h, with periodic (2–3 times) vortexing. After incubation, 75 μl of 8 M KAc were added to the samples, followed by vortexing, incubation on ice for 30 min and centrifugation (10 min at 5,000 × *g* in a microcentrifuge). The supernatant was removed and added to an equal volume of isopropanol, then incubated at –20°C for 1 h. The DNA pellet was recovered by centrifugation, washed with 70% EtOH, then dried and resuspended in 400 μl of filtered water.

DNA from the mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified using primers presented by Howland and Hewitt (1995). Two oligonucleotide primers, 1859S (5′-GAACIGGATGAACTGTTTACCCICC-3′) and 3014A (5′-TCCAATGCACTAATCTGCCATATTA-3′), effectively amplified a single band of the predicted size from *A. tumida* DNA and from the two outgroup taxa. Polymerase chain reaction (PCR) conditions consisted of 94°C for 1 min, 52°C for 1 min and 72°C for 2 min for 35 cycles, followed by a 5-min elongation step at 72°C. Reaction mixtures included 1× PCR buffer (Boehringer Mannheim, Indianapolis, IN), 0.25 μM of each primer, 1 mM dNTP mix and 0.2 U *Taq* DNA polymerase (Boehringer Mannheim). The resulting PCR product was sequenced in both directions by dye-primer sequencing reactions (University of Maryland Center for Agricultural Biotechnology, College Park, MD). Four new primers were designed from the resulting sequence for the *A. tumida.* Primers AT1904S (5′-GGTGGATCTTCAGTTGATTTAGC-3′) and AT2953A (5′-TCAGCTGGGGGATAAAATTG-3′) amplified a 1080-bp region of the COI gene in *A. tumida.* Primer identification numbers refer to the position of these primers (at the 3′ end) with respect to the *Drosophila yakuba* mitochondrial genome (Clary and Wolstenholme 1985). PCR reaction conditions were as above, although with a 60°C annealing temperature instead of 52°C. Two additional primers, AT2380S (5′-GACGTTGATACTCGAGCCTATT-3′) and AT2519A (5′-GAAGTACTCCTGTTA ATCCACC-3′), were used as internal sequencing primers. Sequences were generated for both strands.

Sequence Comparisons and Phylogenetic Analyses. DNA sequences were edited and aligned using the software program Omiga 1.1 (Oxford Molecular Products 1998). Alignment was facilitated by the fact that this coding region contained no insertions or deletions for the taxa studied. Sequences were then exported to the software program PAUP 4.02b (Swofford et al. 1996) for phylogenetic analyses.

The 26 U.S. samples all fell into one of two haplotypes. Accordingly, phylogenetic comparisons were carried out using a single representative from each of these U.S. haplotypes, along with all samples from the South African collections. An exhaustive maximum parsimony search was used to predict phylogenetic relationships among the *A. tumida* samples. Heuristic searches were used for analyses in which hive beetles were rooted with the nitidulid (*Colopterus* sp.) and coccinellid (*Hippodamia* sp.) beetle outgroups. The robustness of individual trees was inferred by the consistency index. Bootstrap analyses were used to infer the strengths of various clades within the trees (n = 10,000 replicates, heuristic branch-swapping search for all analyses).

Table 2. *A. tumida* haplotype counts and frequencies by state

Haplotype	N. Carolina	S. Carolina	Georgia	Florida
NA1	0 (0%)	47 (51%)	1 (6%)	9 (30%)
NA2	12 (100%)	46 (49%)	15 (94%)	21 (70%)
Total	12	93	16	30

Restriction Fragment-Length Polymorphism Analyses. To facilitate future surveys, restriction endonucleases were chosen to distinguish between the two haplotypes common in U.S. hive beetles. PCR products were amplified as above, followed by digestion by one of three diagnostic enzymes (*Alu*I, *Bst*E11, and *Sfc*1) according to suggested buffer and temperature regimes (New England BioLabs, Beverly, MA). Following restriction digests, the products were separated by agarose gel electrophoresis (2% gel, in TAE buffer; 0.04 M Tris-acetate, 0.001 M EDTA). *Alu*I and *Sfc*1 failed to cut the amplified product completely in some samples, and as such provided unclear distinctions between haplotypes. A third enzyme (*Bst*E11, recognition site: GGTCACC) cut only one U.S. haplotype (at position 275), producing an easily scored mobility shift for this haplotype. Accordingly, restriction-site analyses were carried out with enzyme *Bst*E11 on both the sequenced samples and 125 additional beetles.

Results

Genetic Similarity Among *A. tumida* Samples. COI sequences were strongly similar across all of the *A. tumida* samples. The most divergent haplotypes (e.g., CapePtJ2 versus FLVeroB1) differed by only 8 bp (0.8%) across the 1018-bp sequenced region, whereas the average sequence divergence between haplotypes was 0.5% (Table 2). The genetic distance between the two haplotypes found in the United States (6 bp changes) was similar to that found between U.S. beetles and beetles collected in South Africa. Hive beetles differed from the *Colopterus* outgroup taxon by 149 bp (14.5%), on average, and by 221 bp (21%) from the *Hippodamia* sample. These differences are comparable with those found between beetle genera and families, respectively, by Howland and Hewitt (1995). Of the 149 differences between the *Colopterus* sequence and a representative *A. tumida* (SAIxopoA9), 126 (85%) were third-position differences, 20 were first-position and three were second-position differences. Interestingly, there was no bias toward transitions at this level (78 transitions versus 71 transversions), despite the fact that transitions are more likely on the basis of hydrogen bonds between nucleotides (Swofford et al. 1996). This result belies a high overall A/T content at third positions for this locus, such that 55

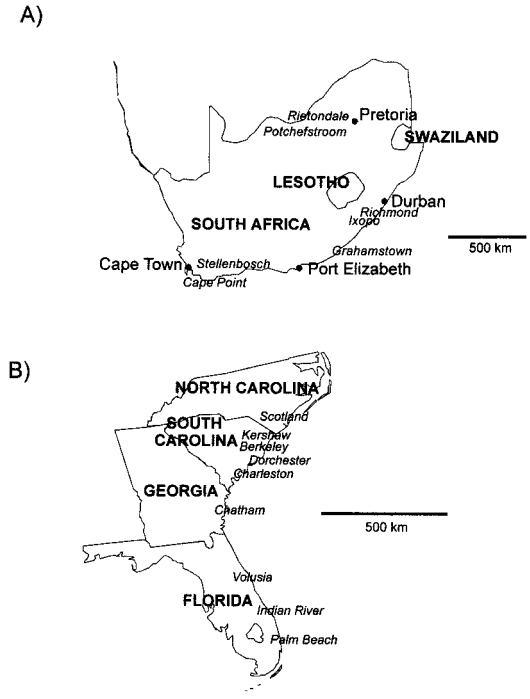

Fig. 1. Map showing collection sites for *A. tumida* from (a) South Africa and (b) the United States. Collection sites shown in italics.

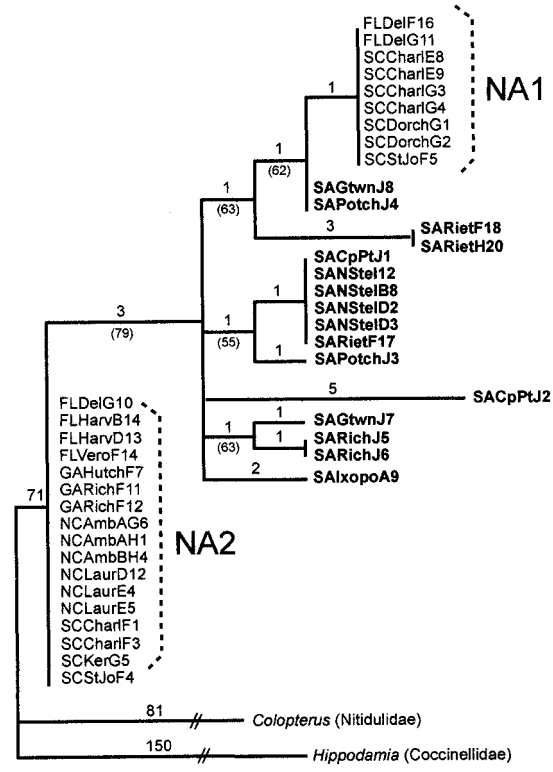

Fig. 2. Phylogenetic relationships between specimens of *A. tumida* and two outgroup taxa, *Colopterus* sp. (Coleoptera: Nitidulidae) and *Hippodamia* sp. (Coleoptera: Coccinellidae). Specimens from South Africa shown in bold. Numbers above line represent branch length, those below line represent bootstrap percentages. Sequences archived in GenBank, accession numbers 227645–227654.

(77%) of the transversions involved an A > T or T > A switch.

Phylogenetic Relationships Among *A. tumida*. The phylogenetic analyses for all hive beetle samples generated a single most-parsimonious tree with a length of 325 steps and a consistency index of 0.985. As shown in Fig. 2, the U.S. hive beetle samples appear to be paraphyletic with respect to samples collected from South Africa. Twenty-two apomorphies were present in the *A. tumida* clade. Of these, 20 occurred in the third (silent) codon position, and one each in the second and third codon position. Twenty of the 22 changes were transitions (A <>G, C <>T), whereas two were transversions (one T > A switch and one T > G switch).

The distinctiveness between the U.S. haplotypes, and between the U.S. and African clades, is supported by bootstrap analyses. One of the U.S. haplotypes (NA1) differed by only a single nucleotide (0.1%) from beetles collected from two sites in South Africa (Grahamstown and Pochefstroom, Fig. 1). The clade that distinguished NA1 from other U.S. hive beetles was supported by bootstrap analyses at the level of 79% (Fig. 2).

Across South Africa, there is little suggestion of a relationship between genetic distance and physical distance on a broad scale, although individuals tended to be more similar within collection sites than across sites. Two South African samples with identical haplotypes were collected from sites separated by several hundred kilometers (Rietondale and Stellenbosch). The number of informative characters at this locus was insufficient for a detailed analysis of genetic structure within Africa.

Variation in Haplotype Frequencies in the United States. Both haplotypes were present in each state surveyed except for North Carolina, where all specimens showed haplotype NA2 (Fig. 2). All of the North Carolina specimens were collected from sites in the Laurinburg region (the first site in which beetles were observed in this state), and this homogeneity could represent a secondary bottleneck among beetles introduced to this area. A majority of the sites surveyed in Georgia, South Carolina, and Florida contained individuals from each of the two haplotypes. The earliest collections made in South Carolina consisted of only haplotype NA1, although sample numbers were small enough that the possibility of missing haplotype NA2 cannot be ruled out (sign test, $P = 0.06$). There was significant heterogeneity, across the four states, in haplotype frequencies (χ^2 test for goodness-of-fit, $P < 0.0001$; Table 2). South Carolina showed nearly equal frequencies of each haplotype, and the remaining

states showed a higher frequency of haplotype NA2 (Table 2).

Discussion

Efforts to control the *A. tumida* will involve a range of strategies, several of which depend on an accurate assessment of the source of beetles present in the United States. Here we present the genetic relationships between *A. tumida* recently introduced in the United States and *A. tumida* found in South Africa. Mitochondrial DNA sequence data strongly suggest that U.S. and South African *A. tumida* represent the same species. Beetles from the United States and South Africa showed mtDNA haplotypes that differed from one another by 0.4%, on average, a range similar to that found across populations in South Africa (0.5%). This degree of divergence is within that found for intraspecific comparisons in other beetle taxa (Vogler et al. 1993, Funk 1999) and in insects generally at the COI locus (e.g., Danforth et al. 1998, Danforth 1999). Further, a fairly deep split, based on mtDNA haplotypes, rendered the U.S. samples paraphyletic with respect to South African *A. tumida.* One U.S. haplotype was nearly identical (1017 of 1018 bp) to beetles collected from two sites in South Africa, and indeed allied more closely with South African samples in the phylogenetic analyses, as indicated by bootstrap values.

Across South Africa, there was some indication of genetic substructuring with respect to mtDNA (Fig. 2), although DNA markers with higher levels of polymorphism are required to quantify this genetic structure. Nevertheless, haplotypes were identical for several widely separated samples (e.g., Rietondale sample F17 and Niev Stellenbosch sample D2), indicating that there is continuing gene flow across and within South African populations. Hive beetles have been identified as associates of honey bees for >60 yr (Lundie 1940) and have likely benefited somewhat from hive movement by beekeepers within Africa. Further, adult *A. tumida* appear to be strong fliers, capable of dispersing over wide distances. Widespread dispersal and gene flow in South Africa suggests that broad-scale containment strategies for the *A. tumida* in North America will be problematic.

Aethina tumida haplotypes in the United States offer insights into the magnitude, and potential source, of introduction(s) of *A. tumida* into the United States. The haplotypic diversity found in the United States, a total of two haplotypes across 26 samples, is substantially lower than that found in South Africa. This paucity of haplotypes suggests either a very limited introduction of hive beetles or subsequent population bottlenecks. With respect to identifying control agents, and minimizing the risk of future introductions, it is important to establish whether the hive beetles currently present in the United States arrived as the result of a single introduction, or two or more introductions. This question remains unresolved. By 1998, both U.S. haplotypes were widespread and intermingled across South Carolina, Georgia, and Florida, a result consistent with a single introduction of beetles which contained individuals from each mtDNA lineage. However, several lines of evidence suggest either two introductions of hive beetles or, as a minimum, irregularities in their transport once beetles arrived in the United States. First, there is significant variance across the states in haplotype frequencies. Recent collections from South Carolina show nearly equal frequencies of each haplotype, even within individual collection sites. In all other states, haplotype NA2 predominates. In fact, this is the sole haplotype found in collections made from North Carolina. Further, early samples from South Carolina consist exclusively of a single haplotype (NA1), whereas haplotype NA2 appeared only in samples collected from 1998 onward. A final suggestion of two distinct introductions comes from the fact that the two haplotypes found in the United States differed substantially from one another at the sequence level. Although some sites in South Africa showed this level of divergence (e.g., at Cape Point), haplotypes tended to be fairly similar within sites. Strong evidence for or against multiple introductions of *A. tumida* into the United States will require sampling using highly polymorphic DNA markers, especially microsatellite DNA (Evans 1993, Queller et al. 1993) and other markers on the nuclear genome. Nevertheless, the mitochondrial DNA polymorphism described here should be helpful for estimating the dispersal of different *A. tumida* populations in time and space in the United States. This information can help predict the mechanisms by which hive beetles invade new hives, locations, and geographic regions in their new range.

Acknowledgments

We especially thank our collaborators for sending samples of *A. tumida* from the United States (J. Ambrose, B. Carter, D. Hopkins, R. Harvey, M. Hood, L. Motes, S. Taber) and from South Africa (M. Allsopp, D. Swart). D. Pham helped with genetic analyses. M. Feldlaufer and two reviewers provided helpful comments on the manuscript.

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Received for publication 23 August 1999; accepted 2 December 1999.